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Ex vivo expansion of hematopoietic stem cells

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Ex vivo expansion of hematopoietic stem cells (HSCs) would benefit clinical applications in several aspects, to improve patient survival, utilize cord blood stem cells for adult applications, and selectively propagate stem cell populations after genetic manipulation. In this review we summarize and discuss recent advances in the culture systems of mouse and human HSCs, which include stroma/HSC co-culture, continuous perfusion and fed-batch cultures, and those supplemented with extrinsic ligands, membrane transportable transcription factors, complement components, protein modification enzymes, metabolites, or small molecule chemicals. Some of the expansion systems have been tested in clinical trials. The optimal condition for *ex vivo* expansion of the primitive and functional human HSCs is still under development. An improved understanding of the mechanisms for HSC cell fate determination and the HSC culture characteristics will guide development of new strategies to overcome difficulties. In the future, development of a combination treatment regimen with agents that enhance self-renewal, block differentiation, and improve homing will be critical. Methods to enhance yields and lower cost during collection and processing should be employed. The employment of an efficient system for *ex vivo* expansion of HSCs will facilitate the further development of novel strategies for cell and gene therapies including genome editing.

***ex vivo* expansion, hematopoietic stem cells, niche, signal transduction, cord blood, transplantation, SCID-repopulating cell, genome editing, CRISPR/Cas9**

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1 Why *ex vivo* expansion of HSCs

Hematopoietic stem cells (HSCs) are defined by their abilities to self-renew and to differentiate into all blood cell types [1–6]. HSCs are the source for all the different lineages of hematopoietic cells and immune cells throughout the human lifetime. Historically, the study of HSCs has been closely related to the potential uses of these cells in clinical applications. HSCs form the basis of bone marrow transplantation and are also a promising cell target for gene therapies [7]. HSC transplantation is used to treat patients with hematopoietic malignancies, genetic defects such as sickle cell anemia and thalassemia, autoimmune diseases, and certain solid cancers [8].

In addition to self-renewal and differentiation, as evidenced by the ability of a single stem cell to repopulate the whole hematopoietic system of a mouse [3,9], HSCs are subject to regulation by apoptosis and migrate in regulated fashion. The balance among various cell fates—quiescence, self-renewal, differentiation, apoptosis, and migration—determines HSC numbers *in vitro* and *in vivo* [10]. Extrinsic modulators (including many cytokines, growth factors, and metabolites) and intrinsic regulators (such as certain transcription factors, cell cycle regulators, and chromatin modulators) control HSC fates through numerous signaling pathways [11–17]. The continued study of the regulation of the HSC fates will further our understanding of stem cell biology and provide important insights into HSC-based

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clinical applications.

A major problem in study and applications of HSCs is the extremely low frequency of HSCs in hematopoietic organs. While the attempt to directly derive differentiated hematopoietic cells from other somatic cells is under development, the techniques to produce clonal, multipotent, and transplantable HSCs from somatic cells, pluripotent embryonic stem cells, or induced pluripotent stem cells prove to be challenging [18,19]. In both autologous transplant and allogeneic transplant, high doses of HSCs are needed to achieve the rapid and sustained engraftment that is critical to patient survival and recovery [20]. Methods to efficiently culture HSCs would further clinical applications in several ways. For example, patients transplanted with larger numbers of stem cells have a better chance of survival, partially due to their successful escape from the host-versus-graft effect [20,21]. In autologous transplantation, some patients do not have sufficient HSCs or need tumor purging, and thus patient HSCs must be expanded *ex vivo*. In addition, umbilical cord blood, a promising cell type for transplantation, does not contain enough cells for adult applications [22,23]. The ability to expand HSCs *ex vivo* would make this enormously important resource useful for adult transplantation. Furthermore, the ability to expand HSCs in culture would greatly boost the development of gene therapy by allowing selection of transducted cells in which the desired gene has been introduced into the appropriate DNA location; this holds the promise for curing a wide variety of human diseases [10]. In particular, the recently developed targeted genome editing techniques including CRISPR/Cas9 technology [24,25] will be greatly benefited by the ability to expand desired manipulated HSCs *ex vivo*.

2 Bone marrow niche of HSCs

The understanding of the extrinsic regulation of HSC fates *in vivo* may provide insights into culture of HSCs. In 1978, the concept of HSC niche was introduced by Schofield [26]. Since then, mounting evidence indicates that the niche plays a crucial role in quiescence, self-renewal, differentiation, apoptosis, migration, and immune privilege of HSCs [27–29]. Several types of cells that potentially form bone marrow HSC niches have been reported [27–30]. The supportive cells in the niches produce growth factors and extracellular matrix components and provide other intercellular signals that promote self-renewal rather than differentiation of HSCs. In the endosteal HSC niche osteoblasts are the main supportive cell type for maintenance of hematopoiesis [31–35]. The vascular HSC niche is mainly composed of perivascular stromal cells and endothelial cells including reticular cells that express stromal cell derived factor 1 (SDF-1) [36], CD146-expressing subendothelial stromal cells [37], Nestin$^+$ mesenchymal stem cells (MSCs) [38], NG2$^+$ periarteriolar cells [39], and perisinusoidal LEPR$^+$ cells [40,41]. In addition, macrophages [42,43], megakaryocytes [44,45], the sympathetic nervous system [46], and adipocytes [47] have also been shown to play roles in the HSC niches. Furthermore, regulatory T cells co-localize with HSCs in the endosteal area of the bone marrow and protect HSCs from immune attack [48]. The clarification of the nature of HSC niche will facilitate to design better strategies for *ex vivo* expansion of HSCs.

3 Expansion of mouse HSCs

Extensive efforts have been made to culture mouse HSCs in two main categories: cytokine cocktail based liquid culture, and stroma/HSC co-culture. The abilities of many cytokines to support hematopoietic progenitors to form colonies *in vitro* provided important insights into expansion of functional primitive long-term (LT-) HSCs that are measured by *in vivo* repopulating activity [49]. In the last two decades, a number of secreted/extracellular proteins/chemicals have been demonstrated to support *ex vivo* expansion of mouse HSCs, including stem cell factor (SCF) [50], thrombopoietin (TPO) [51–53], Notch ligands [54,55], Wnt ligands [56–59], fibroblast growth factor 1 (FGF-1) [60,61], bone morphogenetic proteins (BMPs) [62], Hedgehogs [62–64], prostaglandin E2 (PGE2) [65], interleukin 10 (IL-10) [66], insulin-like growth factor 2 (IGF-2) [67,68], IGF binding protein 2 (IGFBP2) [69,70], several angiopoietin-like proteins (Angptls) [71–74], and pleiotrophin [75]. Conditional derivatives of certain growth factor receptors have also been used to support HSC expansion in culture [76,77]. The introduction of exogenous transcription factors such as homeotic protein HoxB4 can induce dramatic expansion of HSCs [54,56,78–80].

In parallel, the knowledge gained from the co-culture of HSCs with various stromal cell types, including aorta-gonado-mesonephros (AGM), fetal liver, and bone marrow stromal cells, and with endothelial cells and cancer cells has provided important guidance for development of *ex vivo* expansion strategies in medium with defined factors [67,68,71,81–85]. The Williams lab [83] established stromal cell lines from yolk sac that support the activities of HSCs and hematopoietic progenitors. Moore et al. [81,82] isolated a number of stromal cell lines from mouse fetal liver and AGM, and used them to identify HSC-supportive secreted factors. The Rafii group [85] demonstrated that Notch ligands and IGFBP2 produced by endothelial cells support *ex vivo* expansion of mouse HSCs. We identified primary mouse fetal liver stromal cells as a novel HSC supportive population [67,68] and also demonstrated that cancer cells are a rich source of HSC-stimulating proteins [69].

Ex vivo expanded HSCs have gained surprising properties in terms of their interaction with the immune system when transplanted back into mouse recipients. We showed

that *ex vivo*-expanded mouse HSCs dramatically upregulated the cell surface immune inhibitor programmed death-ligand 1 (PD-L1, also known as B7-H1 or CD274) and efficiently repopulated allogeneic recipient mice by overcoming the major histocompatibility complex barrier [73]. In this study, a 40-fold increase of the allograft ability of *ex vivo* expanded HSCs was achieved relative to the uncultured control after 8 d of culture. In addition to the proliferation signal-induced elevation of PD-L1 expression, the increased numbers of functional HSCs also contributed to this enhancement. Our study suggested that extrinsic cues can modulate the immune privilege of HSCs. It also proposed that *ex vivo*-expanded HSCs may lower the matching requirement for allogeneic transplantation and significantly improve the successful rate of this difficult transplantation [73,86–88]. Consistently, *ex vivo* expansion of human HSCs increases regulatory T cell content and decreases the incidence of graft-versus-host disease (GVHD) [89].

4 Expansion of human HSCs

Ex vivo expansion of human HSCs that is important for clinical applications is more challenging than the culture expansion of the mouse counterparts. In early 1990s, the identification of SCF [90–95] and other hematopoietic cytokines [96] led to extensive efforts to culture of human hematopoietic progenitors and HSCs in semi-solid culture and in liquid culture [97–102]. HSCs from human bone marrow, mobilized peripheral blood (mPB), and umbilical cord blood (UCB) have been cultured to expand in a large number of studies. Partially due to their sufficient availability for transplantation and limited transduction ability, there are fewer attempts now to *ex vivo* expand mPB or bone marrow HSCs, which were frequently tried in liquid culture in 1990s to early 2000s [97–99,101–103]. Indeed, the functional mPB HSCs as measured by repopulating activity, were able to be expanded 6-fold after three weeks of culture [104]. Umbilical cord blood, on the other hand, has more proliferative potential and reduced matching requirement and contains more stem cells and results in lower risk of chronic GVHD than mPB does; therefore, cord blood is an attractive source of HSCs [100,105,106]. As a cord blood unit only contains limited numbers of HSCs, it is often not be sufficient for adult transplantation [22,23]. *Ex vivo* expansion would become a straightforward means to enable cord blood cells to be useful in adult applications [22,106–108].

CD34 and CD133 are popular markers to isolate the primitive cord blood cells as the starting populations for HSC expansion [108]. Immune-deficient mice including the severe combined immunodeficiency (SCID), non-obese diabetic (NOD)/SCID, and NOD/SCID IL2Rγ(null) (NSG) mice are popular recipients for transplantation analysis that serves as the "gold standard" to evaluate HSC activity (as SCID-repopulating cells, or SRCs) [109–111]. Although numerous conditions have been used for expansion of HSCs in culture [11,12], a mixture of growth factors/cytokines/chemicals that allows expansion sufficient for clinically applicability has not yet been determined. Below we summarize results from recent attempts to expand human HSCs *ex vivo*. The culture systems range from stroma/HSC co-culture, continuous perfusion and fed-batch systems, and those supplemented with extrinsic ligands, transcription factors, complement components, protein modification enzymes, metabolites, or small molecule chemicals (See below for details, and also see Table 1 at the end of Section 7 with summary of general features and mechanisms of culture system for human cord blood HSCs).

5 HSC-mesenchymal stromal cell co-culture

In vivo, various types of niche cells form a three-dimersion microenvironment for HSCs to control their multiple fates including quiescence, self-renewal, differentiation, apoptosis, and migration. However, under most culture conditions, HSCs undergo apoptosis or differentiation but not self-renewal. In co-culture with primitive hematopoietic cells, various stromal cell types including AGMs, fetal liver, and bone marrow stromal cells, endothelial cells, and mesenchymal stem cells (MSCs) promote HSC expansion [67,68,71,81–85,112,113]. MSCs promote expansion through cell-to-cell contact [114] and cytokine production [115]. Exogenous supplementation or forced expression of HSC-supportive factors in MSCs promotes *ex vivo* expansion of HSCs [116,117]. In a phase I clinical trial [118], a co-culture of HSCs with mesenchymal stromal cells proved to be safe in engraftment and led to an expansion of total nucleated cells and more rapid recovery of neutrophils and platelets than HSCs transplanted without co-culture.

6 Continuous perfusion and fed-batch systems

Although long-term culture of HSCs with cytokines usually leads to differentiation, the removal or dilution of differentiated hematopoietic progenies and their secreted inhibitory signals that negatively regulate HSC self-renewal can promote expansion. A continuous perfusion system [119] and, more recently, a fed-batch system [120] designed to reduce the accumulating negative cues during the culture of HSCs significantly enhanced the expansion of functional primitive HSCs. Both of decreasing the concentration of accumulating negative secreted factors and increasing culture volume to maintain a lower cell density benefits HSC expansion. Using this system, a 12-d culture yielded an 11-fold increase of functional repopulating human cord blood HSCs relative to uncultured controls [120]. The perfusion and fed-batch systems thus represent unique and complementary approaches for expansion of HSCs through regulating the feedback signaling on HSC output.

6.1 Notch ligands

The Notch pathway play major roles in lymphopoiesis, and is also involved in the generation, maintenance, and expansion of HSCs [55]. Notch has a positive effect on self-renewal of HSCs during stressed hematopoiesis but not at steady state [12]. Notch ligands Delta and Jagged support *ex vivo* expansion of HSCs. It is known that activated Notch is capable of immortalizing mouse hematopoietic progenitors with multi-lineage reconstitution ability, and Notch ligands support *ex vivo* expansion of mouse HSCs [12,55]. The effect of Notch ligands on HSCs is dose-dependent: a lower dose of Delta 1 stimulates expansion of human cord blood HSCs, whereas higher amounts of the same factor induce programmed cell death [55]. This scenario seems to be not uncommon when we read the literature about cytokine regulation of cell fates of HSCs. It was suggested that the Notch signaling only supports the *ex vivo* expansion of cord blood HSCs but not adult human HSCs [12]. In a phase I clinical trial, transplantation of immobilized Delta-1-expanded $CD34^+$ human cord blood cells resulted in neutrophil recovery and myeloid engraftment with no signs of GVHD [121].

6.2 Wnts and glycogen synthase kinase 3β (GSK-3β) inhibitor

Wnts are secreted lipidated signaling proteins that bind to Frizzled receptors [122]. Wnt signaling is involved in HSC regulation (reviewed in [59,123]), and Wnt signaling was reported to maintain HSC in a quiescent status *in vivo* [124]. The effects of Wnt signaling are dosage and context dependent: low Wnt doses result in expansion of HSCs, whereas high doses cause exhaustion [125]. Soluble Wnt proteins, including Wnt3a and Wnt5a, support mouse and human HSC activity as determined by repopulation assays [57,58]. It was also reported that *ex vivo* Wnt5a-treated young LT-HSCs decreased HSC repopulation ability [126]. Pretreatment with a GSK-3β inhibitor, which activates the canonical Wnt downstream effector β-catenin, promotes engraftment of *ex vivo*-expanded human HSCs in xenografted mice [127,128].

6.3 Shh/BMP/TGF-β

The inclusion of sonic hedgehog proteins (Shh) in the culture medium of human $CD34^+CD38^-Lin^-$ cells was shown to be able to enhance cell proliferation and increase the repopulation in NOD/SCID recipient mice [71]. As we mentioned earlier, Trowbridge et al. [128] showed that a GSK-3 inhibitor that can modulate multiple pathways including the Hedgehog signaling enhances HSC repopulation.

Shh-induced hematopoietic stem/progenitor cell (HSPC) expansion appears to be dependent on downstream BMP-4 signaling, because inhibition of BMP-4 abrogated Shh-induced expansion [71]. BMPs are members of the transforming growth factor β (TGF-β) superfamily and known to play a critical role in HSC specification during development. BMP signaling negatively regulates the activity of mouse HSCs via control of the endosteal niche [15]. Human HSCs express BMP receptors [72]; the presence of BMP-4 in culture improved the proliferation and maintenance of human HSPCs [62].

A low concentration of TGF-β2 stimulates proliferation of C57BL/6 mouse $Lin^-Sca\text{-}1^+Kit^+$ cells [129,130]. The Karlsson laboratory's work [131] suggested that several TGF-β family ligands induced signaling pathways are intact in mouse HSCs. Pimanda et al. [132] showed the integration of BMP4/Smad signaling in HSC development. The Nakauchi's laboratory [133,134] demonstrated that nonmyelinating Schwann cells produced TGF-β is critical for maintenance of HSC hibernation.

6.4 FGFs

Bone marrow reconstitution analysis demonstrated that an FGF receptor is expressed on all long-term (LT-) mouse bone marrow HSCs [60]. The supplementation of FGF-1 and FGF-2 in serum-free medium of unfractionated mouse bone marrow cells supports the expansion of repopulating HSCs [60,61,135]. Crcareva et al. [135] further demonstrated that the FGF-1 induced expanded HSCs are an excellent source for retroviral gene delivery. Conditional derivatives of FGF receptor-1 have also been used to support short-term HSC expansion and long-term HSC survival in culture [76]. The roles of the FGF pathway in regulating adult HSCs and embryonic hematopoietic development need further investigation. The results from different starting cell populations and under different culture conditions were not all consistent. Schiedlmeier et al. [136] showed that when purified adult mouse HSCs and ES cell-derived HSCs that ectopically express HoxB4 were treated with the fibroblast growth factor receptor (FGFR) inhibitor SU5402 repopulating activity was enhanced. These results indicate the complex nature of the cross-talk between FGF signaling and other pathways, and suggest that FGF regulates HSC activity indirectly [61].

6.5 IGF binding protein 2

IGFBP2 is a member of the IGFBP family that contains at least six circulating proteins binding to IGF-1 and IGF-2 with affinities equal to or greater than those of IGF receptors (IGF-IR, IGF-IIR, and insulin receptor). While IGFBP2 exhibits IGF-dependent inhibitory effects on growth of many types of cells [137], it also displays IGF-independent activities [69,70,138–146]. We identified IGFBP2 from a cancerous cell line as a supportive secreted factor for mouse and human HSCs [69,147]. Concordantly, the Rafii group [85] demonstrated that IGFBP2 is a critical factor secreted by

endothelial cells that supports *ex vivo* expansion of mouse HSCs. IGFBP2 may also support the activity of fetal liver HSCs [148]. These results are accordant with the reported role of nephroblastoma overexpressed (CCN3/NOV), an IGFBP domain-containing protein, which supports expansion of human HSCs [149]. Multiple membrane proteins, including cell surface integrins [139,142–144], Frizzle 8, and low-density lipoprotein (LDL) receptor-related protein 6 [150], were shown to mediate the IGF-independent effects of IGFBP2. Interestingly, extrinsic IGFBP2 can also be taken up into the cytosols of oxidative stressed cells [143,145]. IGF-2 is also a mouse HSC growth factor [67,68]; its effect on human HSCs is unknown. In addition, IGF-2 binds to and stimulates self-renewal of human embryonic stem cells [151]. Despite these data, the mechanisms through which IGFBP2 and IGF-2 support HSC expansion are unclear.

6.6 Angiopoietin-like proteins

Angptls are a family of highly glycosylated secreted proteins that play important roles in metabolism, inflammation, hematopoiesis, and cancer [152,153]. We showed that several Angptls potently stimulate *ex vivo* expansion of bone marrow HSCs [67,68,71]. This result was confirmed by an independent study showing that Angptl3 supports *ex vivo* expansion of mouse HSCs [154], and GST-Angptl5 stimulates *ex vivo* expansion of human cord blood SRCs [147]. In the animal models, Angptls are likely components of the niche of mouse fetal liver and adult HSCs [68,72] and that Angptl1 and 2 are essential to HSC development in zebrafish [155]. We recently showed that leukocyte immunoglobulin-like receptor 2 (LILRB2) is a receptor for multiple Angptls, including GST-Angptl5 [74], and demonstrated that a novel motif in the extracellular domain of LILRB2 mediates Angptl effects [156]. Lin et al. [155] demonstrated that, in human $CD34^+$ cells, Angptl2 induces NOTCH activation via the interaction between LILRB2 and NOTCH, resulting in activation of myc targets.

Because Angptls are large glycosylated proteins that are readily degraded and that form aggregates, these proteins are difficult to express and purify. We developed a serum-free culture system containing defined cytokines and immobilized anti-LILRB2, which supports a 4.9-fold net expansion of repopulating human cord blood HSCs after 10 d of culture, as determined by NSG transplantation [156]. As immobilized antibodies likely prevent internalization of the ligand LILRB2 (that contains the internalization signal YXXphi [157]), receptor activation is prolonged, and thus the *ex vivo* expansion of HSCs can be enhanced by the immobilized antibodies. The anti-LILRB2 polyclonal antibodies are more readily expressed and purified and are more stable than Angptls and, importantly, bind and activate LILRB2 with higher efficiency than Angptl2; use of anti-LILRB2 polyclonal antibodies will have advantages in *ex vivo* HSC expansion systems [156,158].

6.7 Pleiotrophin

Pleiotrophin is a neurite outgrowth factor that is secreted by bone marrow sinusoidal endothelial cells [159]. Pleiotrophin improves the survival of mice following myeloablative treatment [160]. Pleiotrophin supports the *ex vivo* expansion of mouse bone marrow HSCs as determined by competitive repopulating assays and supports *ex vivo* expansion of human cord blood $CD34^+CD38^-Lin^-$ cells as determined by in a SCID-repopulation assay [75]. Pleiotrophin activates phosphoinositide 3-kinase (PI3K) signaling in HSCs, and blocking PI3K or Notch signaling inhibits pleiotrophin-mediated HSC expansion of HSCs [75].

6.8 TAT-HoxB4 and TAT-NF-Ya

The introduction of exogenous transcription factors such as HoxB4 can dramatically expand HSCs [54,56,78–80]. Retroviral overexpression of the human *HOXB4* gene enables dramatic expansion of mouse bone marrow HSCs in culture [79]. A cell-permeable fusion protein TAT-HOXB4 that includes the protein transduction domain of the HIV transactivating protein TAT and HoxB4 expressed by stromal cells in co-culture with human cord blood $CD34^+$ cells for 2 weeks results in 2.5-fold increase in expansion compared to the uncultured controls [161].

NF-Ya, the regulatory subunit of the transcription factor NF-Y, activates *HOXB4* and other genes implicated in the self-renewal and differentiation of HSCs. The inclusion of recombinant TAT-NF-Ya fusion protein in the culture medium of human primary bone marrow cells for 3–9 d results in a 5–10-fold increase of repopulated $huCD45^+$ cells in transplanted NSG mice [162].

6.9 Fucosylating enzymes

An approach to improve the homing and engraftment of human HSCs is to enhance the fucosylation of selectin ligands expressed by these cells. Selectin ligands must be alpha1-3 fucosylated to form glycan determinants such as sialyl Lewis X. Xia et al. [163] showed that insufficient alpha1-3 fucosylation of human cord blood HSPCs led to decreased binding of these cells to E-selectin and P-selectin. The administration of guanosine diphosphate fucose and exogenous alpha1-3 fucosyltransferase VI or VII [163,164] improved the binding of $CD34^+$ cells to selectins. Fucosyltransferase treatment improved homing and the early and long-term engraftment of cord blood $CD34^+$ cells in the bone marrow of immune deficient mice. These results suggest that alpha1-3 fucosylation of HSCs might be critical to homing and engraftment. In a Phase 1/2a clinical trial, a 30 min fucosyltransferase-VI (ASC-101) treatment of human cord blood $CD34^+$ cells in culture improved neutrophil and platelet recovery in engrafted patients [165].

7 Complement component

It was shown that the complement component C3a binds to HSPCs and increases chemokine receptor type 4 (CXCR4) incorporation into membrane lipid rafts to enhance HSPC homing and engraftment [166]. A simple priming of one UCB unit with C3a for 15 min followed by double UCB transplantation was performed in a phase I study [167]. No adverse effects on survival and no infusional toxicities or activation of inflammatory pathways were observed. Engraftment of the C3a-treated UCB, however, was not impaired or favored relative to non-C3a-treated UCB [167].

7.1 CD26/DPPIV inhibitors

Preclinical studies have demonstrated that the expression of peptidase CD26 (also known as dipeptidylpeptidase IV) on donor cells decreases homing and engraftment. By contrast, the inhibition of CD26 increased homing, engraftment, and competitive repopulation of HSCs [168–170]. Based on these findings, a clinical trial was conducted to test whether a CD26/DPPIV inhibitor, Sitagliptin, enhanced engraftment after UCB transplant in adult patients with hematological malignancies. Systemic administration of CD26 inhibitor *in vivo* was safe and may enhance engraftment [171,172].

7.2 Retinoic acid antagonist

Retinoic acid (RA) is part of an effective treatment for acute promyelocytic leukemia; however, the role of retinoid signaling in stem cell biology is not clear. In the mouse system, the activation of the RA pathway by all-*trans* retinoic acid supports *ex vivo* expansion of HSCs [173]. However, when the RA pathway is inhibited by a dominant negative RARα mouse HSC repopulation is also supported [174]. Diethylaminobenzaldehyde, an inhibitor of aldehyde dehydrogenase, the enzyme that is responsible for RA synthesis, induced a 3.4-fold of increase of repopulating cord blood HSCs after 7 d of culture, as determined by SCID repopulation assays [175,176]. Together, these results suggest that RA is an important modulator of HSC homeostasis.

7.3 Copper chelator, TEPA

It was reported that elevated copper concentrations in HSC culture stimulates differentiation. By contrast, the inclusion of tetra-ethylenepentamine (TEPA), a Cu chelator, inhibits maturation and supports expansion of human HSCs as determined by NOD/SCID transplantation [177,178]. In a phase I/II clinical trial, the transplantation ability of TEPA-cultured cord blood cells were tested by co-transplanting with uncultured cord blood cells into patients. The result showed that 90% of patients engrafted with no severe GVHD and a 90% 100-d survival. However, neither neutrophil nor platelet engraftment times were significantly improved [179].

7.4 Epigenetic modifiers

Small molecule inhibitors of histone deacetylase (HDAC) and DNA methyltransferase have been shown to support *ex vivo* expansion of HSCs in multiple studies. The supplementation of human cord blood $CD34^+$ cells in culture with the HDAC inhibitor valproic acid for 7 d upregulated expression of stemness genes, elevated aldehyde dehydrogenase activity, and stimulated a 36-fold increase of SCID-repopulating cells [180]. Another HDAC inhibitor chlamydocin also induced expansion of HSCs in culture [181]. G9a and G9a-like protein (GLP) are methyltransferases that dimethylate histone H3 Lys 9 (H3K9me1/2). Both support hematopoietic lineage specification and differentiation. UNC0638, a G9a/GLP small molecular inhibitor, maintains, but does not induce, expansion of HSC activity in culture [182]. DNA methyltransferase inhibitors and HDAC inhibitors have additive effects in expansion of HSCs in culture. For instance, the DNA methyltransferase inhibitor decitabine [5-aza-2′-deoxycytidine (5azaD)] and the HDAC inhibitor trichostatin A together induced greater ability to maintain HSC activity *in vitro* than individual single agents [183,184]. It was suggested that valproic acid and 5azaD/trichostatin A prevent the loss and support net expansion of HSCs, respectively [185].

7.5 p38 inhibitor

The activation of p38 mitogen-activated protein kinase plays a role in HSC senescence [186,187]. A p38 specific inhibitor, SB203580, supports 3-fold of increase of SRCs after human cord blood $CD133^+$ cells were cultured for 7 d, as determined by the NOD/SCID reconstitution analysis. This effect was primarily attributed to the inhibition of HSC senescence as no significant effect on HSC differentiation and proliferation was observed [188].

7.6 Sirtuin 1 (SIRT1) inhibitor

Nicotinamide (NAM), a form of vitamin B-3, inhibits differentiation and improves homing by inhibiting SIRT1 deacetylase. Treatment of human cord blood HSCs with NAM enhances repopulation in culture [189]. In a phase I study, $CD133^+$ cord blood cells expanded for 21 d in the presence of NAM and the non-cultured $CD133^-$ cells from one UCB unit of cord blood (NiCord) were co-transplanted with an unmanipulated UCB unit into patients with hematologic malignancies. No adverse effects were observed with the infusion of NiCord. NiCord engraftment remained stable in patients, and the patients who received the NAM-treated culture achieved earlier median neutrophil

Table 1 Summary of preclinical and clinical studies of *ex vivo* expansion of human cord blood HSCs

Category of methods	Individual methods	Culture time	Assay	Effects	Mechanism	Reference
Co-culture	HSC-MSC co-culture	14 d	phase I trial	expansion of total nucleated cells and more rapid recovery of neutrophils and platelets	promoting expansion through cell-to-cell contact and cytokine production	[118]
Continuous perfusion	Fed-batch system	12 d	repopulation	11-fold	reducing negative feedback	[119,120]
Cytokine supplememt	Notch ligands	14–21 d	phase I trial	neutrophil recovery and myeloid engraftment with no signs of GVHD	inhibiting differentiation	[121]
	IGFBP2	11 d	repopulation			[147]
	Angptls or anti-LILRB2 antibody	10 d	repopulation	4.9-fold	inhibiting differentiation	[74,147,156]
	Pleiotrophin	7 d	repopulation		activation of the PI3K and Notch pathways	[75]
	TAT-HoxB4	2 week	repopulation	2.5-fold	increasing proliferation and self-renewal of HSCs	[161]
	TAT-NF-Ya	3–9 d	repopulation	4-fold	activating *HOXB4* and other genes implicated in the self-renewal and differentiation of HSCs	[162]
Homing enhancement	Fucosylating enzymes	30 min	phase I/IIa trial	improved neutrophil and platelet recovery	increases homing and engraftment of $CD34^+$ cells	[165]
	Complement (C3a)	15 min	phase I trial	no adverse effect	Increasing homing and engraftment of HSPCs	[167]
Chemical supplement	Retinoic acid antagonsist (diethylaminobenzaldehyde)	7 d	repopulation	3.4-fold	inhibiting differentiation	[175,176]
	Cu chelator (TEPA)	21 d	phase I/II trial	safe	inhibiting differentiation	[179]
	Histone deactylase inhibitor (valproic acid)	7 d	repopulation	36-fold	improving homing and maintaining quiescence	[180]
	DNA Methyltransferase inhibitor (UNC0638)	2 week	repopulation	maintaining HSC activity	blocking formation of higher-order chromatin structure	[182]
	p38 inhibitor	7 d	repopulation	3-fold	inhibiting HSC senescence	[188]
	SIRT1 inhibitor (Nicotinamide)	21 d	phase I trial	earlier median neutrophil recovery	inhibiting differentiation and improving homing	[190]
	AhR antagonist (SR1)		phase I/II trial	enhances neutrophil recovery	antagonizing an acryl hydrocarbon receptor	[191–193]
	PGE2	2 h	phase I trial	enhances neutrophil recovery	enhancing homing, survival, and proliferation of HSCs	[65,194,195]
	TPO receptor agonist (NR-101)	7 d	repopulation	2.9-fold	activating STAT5 and Hif-1alpha	[197]
	UM171	12 d	repopulation	13-fold	inhibiting erythroid and megakaryocytic differentiation	[192]

recovery than those given untreated cord blood [190]. EX-527, another SIRT1 inhibitor, also inhibits differentiation of human $CD34^+$ cells [189]. The mechanism by which NAM supports engraftment of HSCs should be further investigated.

7.7 StemRegenin 1 (SR1)

A purine derivative, StemRegenin 1 (SR1), was identified through a high-throughput screening for the ability to support the *ex vivo* expansion of $CD34^+$ cells in the presence of cytokines [191]. SR1 increases SRC expansion 17 fold. SRI

antagonizes an acryl hydrocarbon receptor (AhR), but how this results in support of HSC expansion is unknown. SR1 does not support *ex vivo* expansion of mouse HSCs or adult human HSCs [182]. Recently, it was suggested that SR1 acts on cells with limited self-renewal potential but that it does not support proliferation of the most primitive HSCs [192]. A phase I/II clinical trial is ongoing and early results indicate that the SR1-containing culture system significantly enhances neutrophil recovery after transplantation [193].

7.8 Prostaglandin E2 (PGE2)

A screening of chemicals that induce HSC proliferation in zebrafish led to the identification of prostaglandin E2 (PGE2) as a stem cell-supportive chemical. Short treatment with PGE2 enhanced *ex vivo* expansion of long-term repopulating mouse HSCs [65] and also supported *ex vivo* expansion of unfractionated and $CD34^+$ cord blood cells as determined by xenograft experiments [194]. PGE2-treated primate $CD34^+$ mPB stem cells exhibit stable multilineage repopulation [194]. A phase I clinical trial with 16,16-dimethyl prostaglandin E2-treated cord blood demonstrated safety and accelerated neutrophil recovery in patients receiving this treatment compared to controls [195].

7.9 Thrombopoietin (TPO) receptor agonist

TPO signaling maintains quiescence and enhances expansion of HSCs during crisis [196]. As a small molecule agonist of the thrombopoietin (TPO) receptor MPL, NR-101 stimulates *ex vivo* expansion of human HSCs [197]. A 2.9-fold increase in SRC numbers was observed in NR-101-treated human $CD34^+$ cells in a 7-d culture compared to uncultured cells, and a 2.3-fold increase was observed compared to human $CD34^+$ cells treated with TPO. NR-101 activates signal transducer and activator of transcription 5 (STAT5) but not STAT3, and also induces activities of HIF-1α and its downstream targets [197].

7.10 UM171

The Sauvageau group [192] screened a chemical library for compounds that support *ex vivo* expansion of mPB $CD34^+CD45R^-$ cells and identified pyrimidoindole derivatives that do not suppress the AhR pathway. An effective compound, UM171, was identified through further modification. In the Fed-Batch culture system [120], UM171 supports expansion of hematopoietic progenitors and results in a 13-fold expansion of SRCs. UM171 cooperates with SR1, an inhibitor of the AhR pathway, to induce an increase of hematopoietic progenitors *in vitro*; UM171, but not SR1, supports expansion of LT-HSCs. Like SR1, UM171 does not have mitogenic activity by itself and thus works together with cytokines. UM171 causes a lymphoid-deficient differentiation pattern in reconstituted mice. Unlike SR1, UM171 inhibits erythroid and megakaryocytic differentiation. The mechanism through which UM171 supports expansion of cord blood LT-HSC expansion and proliferation of adult HSCs or progenitors differs from that of SR1. UM171 is most recently identified compound with HSC stimulatory effects in *ex vivo* expansion culture.

8 Summary and perspectives

The goals of work on *ex vivo* expansion of HSCs are (i) to make one umbilical cord blood unit sufficient for adult transplantation; (ii) to achieve long-term multi-lineage engraftment, reduce the time of neutrophil and platelet engraftment, and facilitate immune reconstitution; (iii) to improve graft efficiency without causing GVHD; and (iiii) to be cost effective. No *ex vivo* expansion protocol has yet achieved these goals. A major problem of the current protocols for *ex vivo* expansion of human HSCs is that there is no convincing evidence that one unit of UCB can be expanded to replace the double units currently required for effective adult transplantation. In some cases, the uncultured UCB unit appears to be responsible for the long-term engraftment. In addition, improved immune reconstitution from expanded UCB units has not been achieved. This may be due to the lack of expansion of primitive human HSCs when existing protocols are used or may result from variation of individual units of cord blood. Moreover, the problem may come from the graft-graft immune reactions of the two transplanted UCB units. To avoid this, the expanded UCB unit may be tried as a sole source for some trials.

Although the optimal condition for *ex vivo* expansion of HSCs is still under development, a better understanding of the mechanisms involved in HSC cell fate determination and the HSC culture characteristics will guide development of new strategies to overcome difficulties. A unique feature of *ex vivo* expansion of HSCs is that no single factor supports HSC expansion. This is possibly due to the fact that HSCs can have different fates—self-renewal, differentiation, apoptosis, and migration. During culture, the cell fates of HSCs are often dominated by differentiation or apoptosis. Therefore, although many factors increase the total cell numbers in HSC culture, in most cases, the cultures are overpopulated by mature differentiated cells rather than the desired most primitive stem cells. Therefore our goal should be to identify factors that support self-renewal and to suppress the other possible cell fates. The most important factors in *ex vivo* expansion of HSCs will thus include factors that inhibit differentiation and apoptosis.

Another interesting observation is that recently identified protein factors and chemicals effective in *ex vivo* expansion of HSCs are not necessarily typical hematopoietic growth factors or those that activate classical HSC pathways. Some newly identified factors induce developmentally conserved

pathways or act on nonessential pathways for HSC function, usually through gain-of-function effects. For example, binding of the receptor LILRB2 by the ligand Angptls supports HSC expansion; however, LILRB knockout in mice does not have an overt phenotype in hematopoiesis [74]. The AhR pathway is non-essential during HSC development but appears to be important for *ex vivo* expansion of HSCs as demonstrated by the effectiveness of the AhR antagonist SR1 [191].

An unresolved question that limits the future development of improved systems for *ex vivo* expansion of HSCs is why the surface phenotype of cultured HSCs differs so dramatically from that of freshly isolated HSCs. Therefore there is no reliable *in vitro* measure of the activity of cultured HSCs. It is well aware that the surface phenotypes of mouse and human HSCs are changed upon *ex vivo* culture [84,198,199]. Due to a lack of better markers, $CD34^+CD38^-CD90^+CD45R^-CD49f^+$, the surface phenotype of freshly isolated human HSCs [200] has been used to screen for HSC supportive chemicals *in vitro* [192]. Better *in vitro* measures of HSC activity would make screening more effective.

The cost of *ex vivo* culture of HSCs is relatively high. It is an open question whether we should use enriched or unenriched HSCs as a starting population for *ex vivo* expansion. Most currently used *ex vivo* expansion approaches use partially enriched HSCs such as $CD34^+$ cells or $CD133^+$ cells. These cells appear to expand more readily than unenriched cells—at least in terms of number increases. Nevertheless, a portion of HSCs present in the original unfrozen cord blood unit may be lost during this fractionation process. Methods to minimize processing or optimize enrichment should be identified.

In the future, work should proceed in several directions to improve the existing systems for *ex vivo* expansion of HSCs. First, new approaches to enable expansion of the primitive multi-lineage HSCs—not only the progenitors—are essential. Second, a combination of approaches are necessary to optimally expand HSCs. These might include development of a combination treatment regimen with agents that enhance self-renewal, that block differentiation, and that improve homing. For instance, given the interaction between Notch and Angptl2 receptor in human HSPCs [155], it is reasonable to propose to combine the Notch ligands- and Angptl-based HSC culture systems for further improvement of the *ex vivo* expansion of primitive cord blood HSCs. In addition, 3D culture and fed-batch culture methods may be pursued, and methods to enhance yields and lower cost during collection and processing should be developed. Furthermore, a cord blood bank would be useful if pre-expanded units were in stock. In this way, sufficient numbers of functional stem cells from the expanded cord blood HSCs can be directly transplanted into matched adult patients. Finally, the application of the targeted genome editing techniques to HSCs can be limited without the capacity of selective expansion of the HSCs in which the desired genomic loci are modified [201]. A combination of techniques in *ex vivo* expansion and genome editing such as CRISPR/Cas9 technology would enable the further development of HSC-based gene therapy.

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